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### Authors

Ribalet, B  
Ciani, S  
Eddlestone, GT

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# ATP Mediates Both Activation and Inhibition of K(ATP) Channel Activity via cAMP-dependent Protein Kinase in Insulin-secreting Cell Lines

B. RIBALET, S. CIANI, and G. T. EDDLESTONE

From the Department of Physiology, Ahmanson Laboratory of Neurobiology, Brain Research Institute and Jerry Lewis Neuromuscular Research Center, University of California, Los Angeles, Los Angeles, California 90024

**ABSTRACT** The single-channel recording technique was employed to investigate the mechanism conferring ATP sensitivity to a metabolite-sensitive K channel in insulin-secreting cells. ATP stimulated channel activity in the 0–10  $\mu$ M range, but depressed it at higher concentrations. In inside-out patches, addition of the cAMP-dependent protein kinase inhibitor (PKI) reduced channel activity, suggesting that the stimulatory effect of ATP occurs via cAMP-dependent protein kinase-mediated phosphorylation. Raising ATP between 10 and 500  $\mu$ M in the presence of exogenous PKI progressively reduced the channel activity; it is proposed that this inactivation results from a reduction in kinase activity owing to an ATP-dependent binding of PKI or a protein with similar inhibitory properties to the kinase. A model describing the effects of ATP was developed, incorporating these two separate roles for the nucleotide. Assuming that the efficacy of ATP in controlling the channel activity depends upon the relative concentrations of inhibitor and catalytic subunit associated with the membrane, our model predicts that the channel sensitivity to ATP will vary when the ratio of these two modulators is altered. Based upon this, it is shown that the apparent discrepancy existing between the sensitivity of the channel to low ATP concentrations in the excised patch and the elevated intracellular level of ATP may be explained by postulating a change in the inhibitor/kinase ratio from 1:1 to 3:2 owing to the loss of protein kinase after patch excision. At a low concentration of ATP (10–20  $\mu$ M), a nonhydrolyzable ATP analogue, AMP-PNP, enhanced the channel activity when present below 10  $\mu$ M, whereas the analogue blocked the channel activity at higher concentrations. It is postulated that AMP-PNP inhibits the formation of the kinase–inhibitor complex in the former case, and prevents phosphate transfer in the latter. A similar mechanism would explain the interaction between ATP and ADP which is characterized by enhanced activity at low ADP concentrations and blocking at higher concentrations.

Address reprint requests to Dr. B. Ribalet, Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90024.

## INTRODUCTION

It is generally accepted that the resting membrane potential of the pancreatic B cell is maintained by a K channel modulated by glucose, increasing glucose in the range of from 0 to 5–7 mM causing progressive inhibition of channel activity (Misler et al., 1986; Ashcroft et al., 1988; Ribalet et al., 1988). A decrease in membrane K permeability and concomitant cell–membrane depolarization trigger the characteristic oscillatory electrical pattern associated with insulin secretion (Atwater et al., 1978). The glucose-sensitive channel has been identified with a channel whose activity in isolated membrane patches from pancreatic B cells as well as cardiac and skeletal muscle is inhibited when ATP is present at the intracellular face of the membrane (Cook and Hales, 1984; Kakei and Noma, 1984; Spruce et al., 1987). We shall refer to this channel as the K(ATP) channel. Since B-cell ATP content rises dose dependently as extracellular glucose is elevated in the range 0–7 mM (Ashcroft et al., 1973; Malaisse et al., 1979), it has been hypothesized that changes in the intracellular concentration of the nucleotide mediate the effect of glucose on channel activity. While measurements of B-cell mean intracellular ATP concentration vary in the range 3–6 mM (Ashcroft et al., 1973; Malaisse et al., 1979), half-maximal channel inhibition occurs between 10 and 50  $\mu$ M in excised patches; since channel activity is observed in the cell-attached patch, it must be concluded that, in this configuration, either the ATP level close to the channel is lower than the average cellular concentration, or that additional channel modulators are present. In pursuit of this second possibility several studies have reported that in excised patches ADP antagonises the effect of ATP, permitting activity to be recorded at levels of ATP significantly greater than 1 mM (Dunne and Petersen, 1986b; Kakei et al., 1986; Misler et al., 1986; Ribalet and Ciani, 1987). The present study incorporates these observations while providing a mechanistic basis to explain the phenomenon of ATP-sensitive K channel activity modulation.

Since nonphosphorylating ATP analogues (e.g., AMP-PNP, AMP-PCP) inhibit the K(ATP) channel in the inside-out patch, it is generally accepted that channel inhibition by ATP is not mediated by phosphorylation (Cook and Hales, 1984). However, protein phosphorylation has often been evoked to explain the observation that a minimal level of ATP is required to prevent a rapid decrease of channel activity after patch excision, a phenomenon referred to as channel “run-down” (Findlay and Dunne, 1986; Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987; Ashcroft, 1988).

The studies detailed below were undertaken to explore the possible role of protein phosphorylation in modulation of the K(ATP) channel. The results obtained in this investigation provide indications that molecules of the catalytic subunit of the cAMP-dependent protein kinase (protein kinase A) are usually present in isolated patches from insulin-secreting cells, and that phosphorylation, promoted by that enzyme, is capable of increasing the activity of the K(ATP) channel.

The mechanism of action of protein kinase A is fairly well characterized; the holoenzyme, comprising two catalytic and two regulatory subunits, must be dissociated by cAMP before the enzyme becomes active. Since cAMP in insulin-secreting cells is believed to either remain constant or increase in the presence of glucose (Sharp, 1979), channel modulation by protein kinase A alone could not effect chan-

nel closure. However, it has been suggested that, despite the presence of elevated concentrations of cAMP, the activity of protein kinase A may be low in unstimulated cells as a result of the interaction between the catalytic subunit and a specific inhibitory peptide (protein kinase inhibitor [PKI] or Walsh inhibitor) which competes for the substrate binding site (Ashby and Walsh, 1972; Beavo et al., 1974). Of central significance in this investigation of an ATP-dependent K channel is the recent finding in biochemical studies that ATP binding to the catalytic subunit of protein kinase A enhances the interaction of the latter with PKI, thereby reducing the ability of the enzyme to phosphorylate target proteins (Whitehouse et al., 1983; Whitehouse and Walsh, 1983). This led us to speculate that the role of ATP in reducing the activity of the K(ATP) channel may reside in its ability to facilitate the interaction between protein kinase A and the PKI, leading to a reduction in phosphorylation and channel closure. In support of this we find that exogenous addition of the inhibitor to the "intracellular" solution of an isolated patch reduces the activity of the K(ATP) channel, the effect becoming increasingly pronounced as the ATP concentration is raised.

On the basis of hypotheses suggested both by our data and the experimental background summarized above, we present a simple model, showing that the effects of ATP on K(ATP) channel activity can be described by postulating a dual role for the nucleotide: one as phosphate source for the reaction catalysed by protein kinase A, promoting channel opening, the other as mediator of enzyme inhibition by favoring the interaction between the catalytic subunit of protein kinase A and the peptide inhibitor, promoting dephosphorylation and channel closure.

Biochemical studies have demonstrated the presence of the inhibitor in pancreatic cells (Kuo, 1975), however since there is no evidence that it is bound to the B cell membrane, other mechanisms that could also cause protein dephosphorylation will be considered in the Discussion.

## MATERIALS AND METHODS

### *Cell Cultures and Experimental Media*

Experiments were performed using cells from the insulin-secreting cell lines RINm5F and HIT (both kindly provided by A. E. Boyd III of Baylor College of Medicine). The cells were incubated at 37°C in RPMI 1640 medium, supplemented with 10% (vol/vol) fetal calf serum/penicillin (100 U/ml)/streptomycin (100 U/ml)/2 mM glutamine. The cells were divided once a week by treatment with trypsin and the medium was changed twice between two successive divisions. At 1 h before starting the experiment the culture medium was exchanged with a glucose-free solution containing 135 mM NaCl, 5 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.1 mM MgCl<sub>2</sub>, and 10 mM Hepes, the pH being adjusted to 7.2 with NaOH. The composition of the patch pipette solution was 140 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.1 mM MgCl<sub>2</sub> and 10 mM Hepes, pH being adjusted to 7.2 with KOH; that of the bath solution, for experiments with excised inside-out patches, was similar, except that no calcium was added. ATP (Sigma Chemical Co., St. Louis, MO) and AMP-PNP (Boehringer Mannheim Diagnostics, Inc., Houston, TX) were added to 10 ml of the bath solution that was circulated through a 1-ml experimental chamber. Before the experiments the proteins (enzyme and inhibitor), were solubilized in 100 µl of bath solution. This concentrated solution was diluted by directly adding appropriate aliquots to the experimental chamber. Two different samples of the catalytic subunit of the cAMP-

dependent protein kinase were used: one was the commercially available product from Sigma Chemical Co.; the other was a sample purified in E. Fisher's laboratory (University of Washington). The PKI was also purchased from Sigma Chemical Co.; the synthesized inhibitor (PKI 5-24, amide) was obtained from Peninsula Laboratories Inc., Belmont, CA.

#### *Data Recording and Analysis*

The techniques to prepare patch electrodes and record single-channel events were similar to those we have described before (Ribalet and Ciani, 1987), as well as similar to those presented in great detail by Hamill et al. (1981). The data, filtered at 2 kHz with an eight-pole Bessel filter, and recorded either with an EPC 7 List (Darmstadt, Federal Republic of Germany) or an Axopatch 1A (Axon Instruments, Inc., Burlingame, CA) patch clamp amplifier, were then digitized with a digital audio processor and stored on video cassette tape at a fixed frequency of 44 kHz. For analysis, the data were acquired on an IBM AT hard disk at a rate of 5.5 kHz, the transfer being performed with a two-buffer interface allowing continuous acquisition (Bezanilla, 1985). Prior to analysis of the channel open and closed time distributions, amplitude histograms of the current steps were built to determine the half amplitude threshold, and this threshold was used to form an idealized record of the original data (Colquhoun and Sigworth, 1983). This schematized record was utilized for the subsequent analysis. The percentage open time, which is the parameter used to assess the level of channel activity, was determined from data samples of 1-min duration. For the construction of the open and closed time histograms, longer stretches of data (2–4 min) were often necessary, especially to obtain adequate resolution for determining the distribution of the long closed-time intervals. The histograms were constructed using bin sizes that were multiples of the acquisition rate. For the open time intervals, the histograms were built employing bin sizes of either 180 or 360  $\mu$ s, which were then fitted with a sum of two exponentials using a least square algorithm. For the closed time intervals, where bins of either 11.5 or 23 ms were used, two exponentials were required again for an adequate fitting. However, in the closed-time histograms, the first point contained a large number of events, which were not included in the theoretical curve. This group of short-lasting events was replotted, using a smaller bin-size (180  $\mu$ s) for adequate resolution, and the resulting distribution was fitted with a single exponential. In summary, three exponentials were needed for the closed-time, and two for the open-time distributions.

### RESULTS

#### *Effects of Noninhibitory ATP Concentrations on K(ATP) Channel Activity*

In cell-attached patches the ATP-dependent K channel openings rarely exceed a single level, however, after patch excision, the properties of the activity change considerably. Initially the activity increases, with multiple levels of current being often observed, as shown in Fig. 1 (*upper panel*), where three current levels were recorded. This is always followed by a progressive decline of the activity, an effect referred to as channel "run-down." The lower panel of Fig. 1 illustrates that addition of a low concentration of ATP (10  $\mu$ M) soon after excision of the patch reversed channel "run-down," this effect of ATP required the presence of  $Mg^{2+}$  in the medium. Under these conditions channel activity could be maintained for several hours though at a level generally lower than observed immediately after excision. In contrast, addition of the nucleotide 10–15 min after the activity had ceased, failed to reactivate the channel (result not shown). Similar phenomena have been observed

with B cells from primary culture (Misler et al., 1986; Ashcroft, 1988). On the basis of these observations, it has been surmised that phosphorylation, either of the channel or of a closely associated protein, is implicated in maintaining channel activity (Findlay and Dunne, 1986; Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987; Ashcroft, 1988).

#### *Investigation of the K(ATP) Channel Phosphorylation*

Using the cell-attached patch configuration, a membrane permeant analogue of cAMP, dibutyryl cAMP, was added to the medium to test whether activation of endogenous cAMP-dependent protein kinase (protein kinase A) influenced K(ATP) channel kinetics. Fig. 2 A (*upper trace*) illustrates the stimulatory effect of 50  $\mu$ M dibutyryl cAMP on channel activity; the activity increased slowly until a new steady

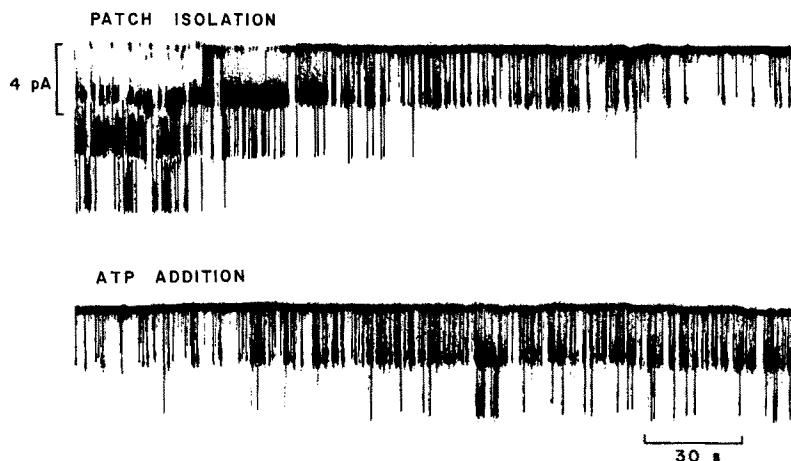


FIGURE 1. The upper trace illustrates the “run-down” of K(ATP) channel activity in an excised inside-out patch exposed to a 140 mM  $K^+$  solution with no ATP added. The patch was isolated  $\sim$ 15–20 s before the beginning of the trace. The lower trace illustrates the substantial restoration of activity which follows addition of 15  $\mu$ M ATP. The record begins at the end of the 30-s perfusion period during which ATP was added.

state was reached  $\sim$ 7 min after nucleotide addition. In four experiments the observed increase of activity averaged 150%. The fact that dibutyryl cAMP stimulated the K(ATP) channel indicated a role for protein kinase A in modulation of this channel.

Since activation of cAMP-dependent protein kinase affects several cellular functions, it was necessary to test more directly the role of the enzyme in K(ATP) channel regulation. Thus, experiments were carried out with excised inside-out patches, exposing the intracellular side of the membrane to solutions containing the catalytic subunit of protein kinase A in the presence of noninhibitory levels of MgATP (10–20  $\mu$ M). The lower trace of Fig. 2 A illustrates the effect of 0.25  $\mu$ M catalytic subunit on channel activity; a progressive stimulation was observed which required 7–10 min to reach a new steady state. In seven experiments a mean increase of activity of

about 230% occurred after addition of the enzyme. Catalytic subunit of the kinase from two sources was used (see Materials and Methods), their effects were not noticeably different.

Although these experiments indicated that phosphorylation by exogenously added kinase modulates channel activity, it remained to be demonstrated that a native enzyme was associated with the channel protein which could similarly affect its activity. To investigate this possibility a specific peptide inhibitor of cAMP-depen-

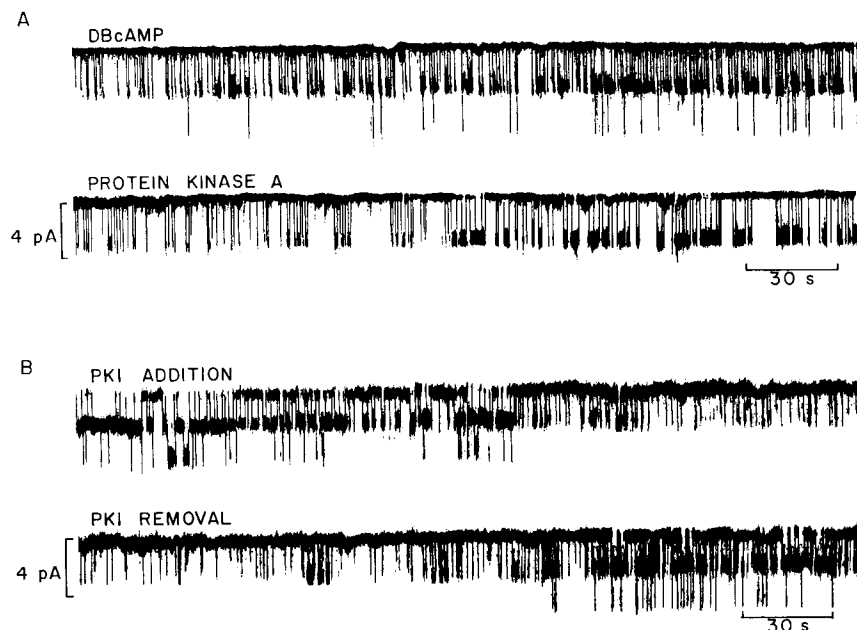


FIGURE 2. (A) The upper trace displays the time course of K(ATP) channel activity in a cell-attached patch after addition of 50  $\mu$ M dibutyryl-cAMP (completed at the left end of the trace) to a normal extracellular solution. In the lower record, the time course of K(ATP) channel activity is shown in an isolated inside-out patch, after addition of 0.25  $\mu$ M of the catalytic subunit of the cAMP-dependent protein kinase to a solution containing high  $K^+$  (140 mM) and 10  $\mu$ M ATP. (B) K(ATP) channel activity in an excised inside-out patch, after addition of 10  $\mu$ M of a specific inhibitor of the cAMP-dependent protein kinase (PKI). When the synthesized inhibitor was used inhibition of the activity could be observed with concentrations as low as 5  $\mu$ M. The solution was the same as in the lower record of Fig. 2 A, but in this experiment 50  $\mu$ M ATP was present. The lower record shows recovery of channel activity in the same patch after removal of the inhibitor.

dent protein kinase was used. Fig. 2 B illustrates a representative inside-out patch experiment in which this inhibitor was added to the bath at a concentration of 10  $\mu$ M; in eight similar experiments a concentration range of 2–10  $\mu$ M was used in the presence of 50  $\mu$ M MgATP. After addition of the inhibitor to the bath a progressive decline in channel activity was observed, lasting for 5–10 min before stabilizing at a new steady state. Since no exogenous kinase was added, the reduction in activity seen in Fig. 2 B must be attributed to an interaction between the inhibitor and a

native kinase. Similar inhibitory effects were produced both by the protein kinase inhibitor isolated originally by Ashby and Walsh (1972), and by a shorter synthetic peptide, identified as the functional component for binding to the catalytic subunit of the enzyme (Scott et al., 1985; Cheng et al., 1986). The lower trace in Fig. 2 *B* illustrates that removal of the kinase inhibitor was followed by a rapid reversal of its effect.

*Effects of Inhibitory Concentrations of ATP and of PKI on K(ATP) Channel Activity*

The blocking effect of ATP on K channels was first described in cardiac cells by Noma (1983), later in pancreatic B cells from neonatal rats by Cook and Hales (1984), and in skeletal muscle by Spruce et al. (1985). Since then, blockage of chan-

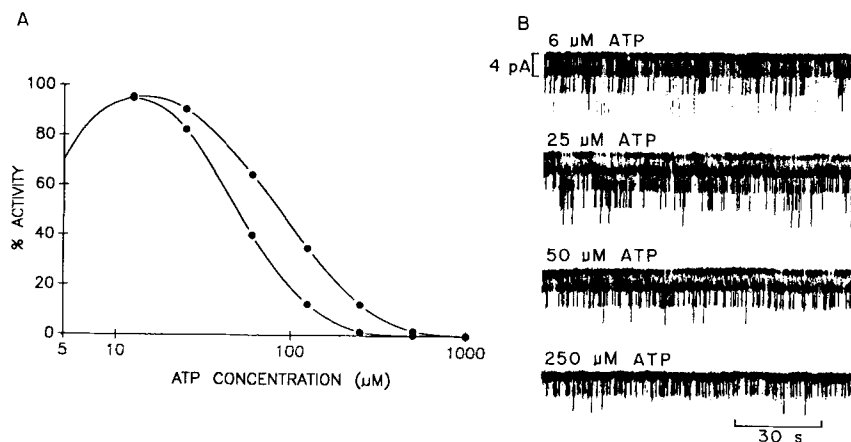


FIGURE 3. (A) The two sets of points illustrate the degree of variability of ATP sensitivity of K(ATP) channel activity in different excised inside-out patches. The activities, normalized to their maximum value, are defined as the area subtended by the open-state outline of the recording trace for a given time (10–15 s). (B) Record traces of steady-state K(ATP) channel activity taken from a representative inside-out patch exposed to different ATP concentrations. Note that the activity increased with ATP level from 6–25  $\mu$ M, but decreased thereafter.

nel activity by ATP has been reported frequently in B cell lines derived from insulinoma, as well as in B cells of normal pancreatic tissues from rats (Rorsman and Trube, 1985; Dunne et al., 1986; Kakei et al., 1986; Misler et al., 1986; Ribalet and Ciani, 1987; Ashcroft, 1988).

Fig. 3 shows the effect of ATP on channel activity in patches excised from cells of the RINm5F cell line. Although there was some variability in channel ATP sensitivity in different patches, increasing ATP up to 10–20  $\mu$ M generally stimulated K(ATP) channel activity in both the RINm5F and HIT cells, but depressed it thereafter, complete inhibition being attained when the concentration of the nucleotide was of the order of 0.5–1 mM. Two sets of points (empirically fitted by continuous curves) are exhibited in Fig. 3 *A* to illustrate the variability in the sensitivity to ATP observed with different patches. The half-maximal concentrations in these extreme examples



are  $\sim 50$  and  $85 \mu\text{M}$ , values significantly higher than that found in neonatal rats (Cook and Hales, 1984), but similar to values reported for these cells (Ribalet and Ciani, 1987), and for HIT cells (unpublished results). Representative data are illustrated in Fig. 3 *B*, where it is clear that the activity recorded in the presence of  $25 \mu\text{M}$  ATP was larger than at  $6 \mu\text{M}$ , while at concentrations above  $25 \mu\text{M}$  the activity diminished as the level of nucleotide was increased.

Since we had shown that PKI reduces channel activity, and it had previously been demonstrated by Whitehouse and Walsh (1983) that ATP enhanced the binding of

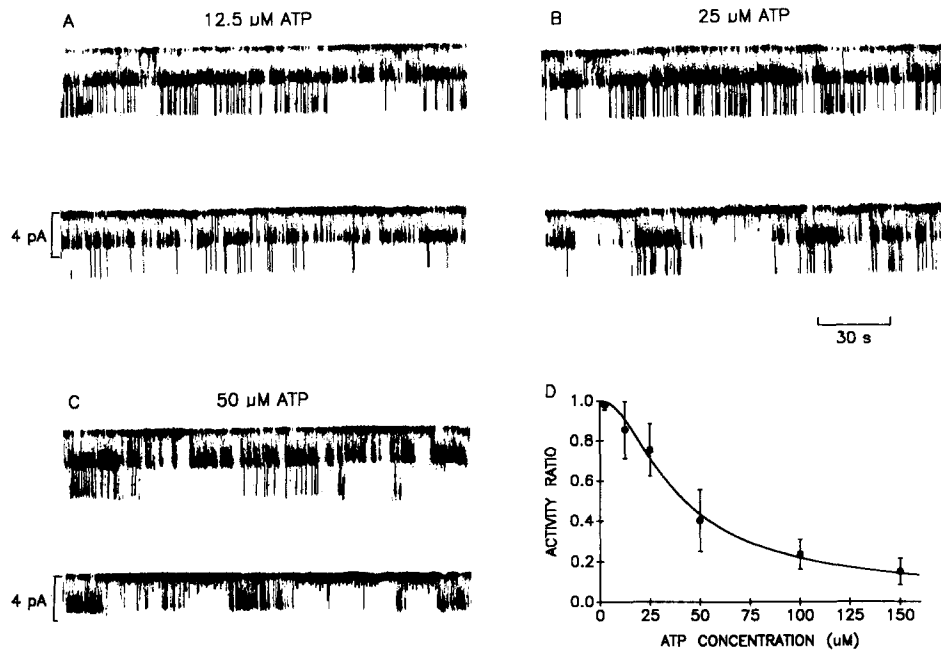


FIGURE 4. Effects on K(ATP) channel activity in excised inside-out patches of exogenously added PKI to solutions with different ATP concentrations. For each of the three pairs of records, *A*, *B*, and *C*, the data in the upper trace were obtained in the absence of the inhibitor, in the lower trace the data were obtained in the presence of the inhibitor at  $15 \mu\text{M}$ . All the records correspond to experiments with the same patch. In *D*, the activity ratio (defined as the ratio of the activity in the presence of the inhibitor to that in its absence) is plotted as a function of the concentration of ATP. The continuous curve was generated by fitting the data with the model presented in this paper.

this inhibitor to the catalytic subunit of protein kinase A, we hypothesized that the blocking effect of ATP was mediated via a mechanism involving this inhibitor. To test this possibility, the effect of the inhibitor was investigated as a function of ATP as shown in Fig. 4. The three paired records in *A*, *B*, and *C* show, for the ATP concentrations indicated, the channel activities in the absence (*upper lines*) and in the presence (*lower lines*) of the inhibitor. The ATP dependence of the inhibition is illustrated in *D*. This plot of the ratio of the activity in the presence to that in the absence of the inhibitor, at each concentration of ATP, clearly shows that the block-

ing efficacy of the PKI is enhanced by the nucleotide. The continuous line in this figure as well as those in Figs. 6–8, were deduced from a theoretical fit with the model described in the section after the next.

#### *Effects of ATP and PKI on Channel Kinetics*

Inspection of the data illustrated in Fig. 4 indicated that the PKI emphasized the burstlike pattern of the channel activity, an effect already observed with ATP (Kakei and Noma, 1984; Spruce et al., 1987; Ashcroft, 1988) (see also Table I). For this reason kinetic analysis of the data in terms of open- and closed-time histograms was performed to investigate which kinetic parameters (time constants in this type of analysis) were affected by the inhibitor and by ATP. Two exponentials were needed to fit the open-time histogram and three to fit the close-time histogram. Fig. 5 and Table I illustrate the effect of the inhibitor and of ATP on the various time constants. In Fig. 5, which refers to experiments at constant ATP levels (25  $\mu$ M), the

TABLE I  
*Effects of ATP and PKI on Two of the Time Constants of the Open- and Closed-Time Histograms*

ATP $\mu$ M		Without inhibitor	With inhibitor
6	$t_2$ (open)	1.56	—
	$t_3$ (closed)	86.30	—
12.5	$t_2$ (open)	2.21	$2.40 \pm 0.29$
	$t_3$ (closed)	77.00	$92.50 \pm 18.42$
25	$t_2$ (open)	$1.97 \pm 0.24$	$2.00 \pm 0.42$
	$t_3$ (closed)	$75.60 \pm 19.00$	$180.90 \pm 27.8$
50	$t_2$ (open)	$1.96 \pm 0.15$	$2.02 \pm 0.35$
	$t_3$ (closed)	$101.33 \pm 15.80$	$232.40 \pm 60.5$
100	$t_2$ (open)	$2.15 \pm 0.12$	$1.97 \pm 0.44$
	$t_3$ (closed)	$123.60 \pm 25.30$	358.00
200	$t_2$ (open)	2.16	—
	$t_3$ (closed)	158.00	—

histograms in the upper panel were derived from data obtained without inhibitor, those in the lower panel from data with inhibitor. The two time constants of the open-time histogram (shown in *A1* and *B1*), as well as the two fastest time constants of the closed-time histogram (*A2*, *A3*, and *B2*, *B3*), were little affected by the inhibitor. In contrast, addition of the protein inhibitor provoked a twofold increase in the slowest time constant of the closed-time histogram (*A3* and *B3*).

Derivation of the time constants from open- and closed-time histograms from data recorded at different ATP concentrations indicated that ATP similarly affects just this one time constant. Table I summarizes experimental data at different ATP concentrations in the presence and absence of the protein kinase inhibitor. For simplicity, since the only time constant affected by either ATP or the kinase inhibitor was the slow time constant of the closed-time histogram, this value is given [ $t_{3(\text{closed})}$ ] along with one of the remaining four [ $t_{2(\text{open})}$ ], the latter to illustrate the lack of effect of ATP and PKI.

These data illustrate the qualitatively similar effects of ATP and PKI on the channel kinetics. This type of finding suggests that ATP and the inhibitor are elements of a common modulatory pathway. The model elaborated in the next section makes use of a similar proposal.

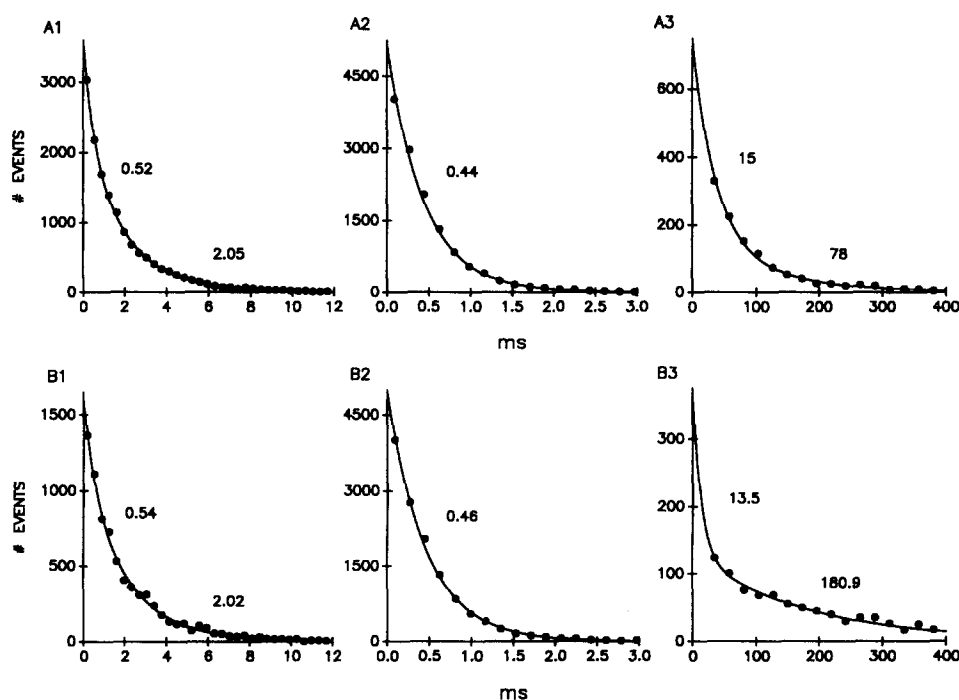


FIGURE 5. Effects of PKI on the distribution of open and closed times for the K(ATP) channel in excised inside-out patches exposed to solutions with 25  $\mu$ M ATP. All the histograms were calculated from records in which only one step of current was present. In A1, A2, and A3, the inhibitor was absent; in B1, B2, and B3, it was present at 15  $\mu$ M. A1 and B1, which are fitted with two exponentials, represent the open times distribution. The fitting of the closed time distribution required, within our limits of resolution, at least three exponentials. A2 and B2 indicate a short closed time, probably corresponding to the closed times within bursts; A3 and B3 indicate two longer closed times, probably corresponding to the silent periods between bursts. The histograms indicate that only the time constants for the exponentials pertaining to the distributions of the long closed times are significantly affected by the inhibitor.

#### *Model for the Role of Phosphorylation in the K(ATP) Channel Activity*

The model proposed is based on the premise, suggested by our data, that opening of the K(ATP) channel is promoted by phosphorylation via a cAMP-dependent protein kinase. The channel will be referred to as the phosphate-accepting molecule and it will be assumed that the activity is proportional to the percentage of phosphorylated channels in the patch. According to this viewpoint the various steady levels of activity at different ATP concentrations would reflect the attainment of

stationary rates for a cascade of reactions, corresponding to different fractions of phosphorylated channels in the patch. However, if only one channel is present in the patch, the various steady states of activity would correspond to different probabilities that the single channel is phosphorylated. Since we do not know all the reactions involved, a complete description of the kinetics will be impossible, and therefore the scope of the model will be to derive testable predictions for the channel activity as a function of controllable parameters, such as the concentrations of ATP and AMP-PNP (a nonhydrolyzable ATP analogue).

Postulating that standard concepts of enzyme kinetics can be used in the microenvironment of the patch, the rate of phosphorylation will be considered proportional to the number of enzyme-substrate complexes in the patch, which we denote by [EAS]. E stands for the uncomplexed enzyme (kinase), A for ATP, and S for the phosphate-accepting substrate. If only one EAS complex is present in the patch, either because there is only one channel or/and, only one enzyme molecule, the quantity [EAS] represents the probability that enzyme, channel, and ATP will aggregate to form one EAS complex. It will also be assumed that the rate of channel dephosphorylation, via an appropriate phosphatase, can be considered proportional to the number of products of phosphorylation, namely to the number of phosphorylated channels in the patch, [AS]. Thus, at steady state we shall have

$$\frac{d[AS]}{dt} = k_p[EAS] - k_D[AS] = 0, \quad \text{or} \quad [AS] = \frac{k_p}{k_D} [EAS], \quad (1)$$

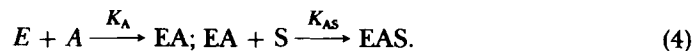
where  $k_p$  and  $k_D$  are the rate constants for phosphorylation and dephosphorylation. Since the channel activity (denoted briefly as [ch. act.]) is assumed proportional to the number of phosphorylated channels, [AS], we can write, recalling the second of the Eqs. (1):

$$[\text{ch. act.}] = P [EAS], \quad (2)$$

$P$  being a proportionality factor that incorporates the ratio  $k_p/k_D$ . Assuming that the number of EAS complexes is in equilibrium with its molecular components, we will have

$$[EAS] = K_A K_{AS} [E][A][S], \quad (3)$$

where  $K_A$  and  $K_{AS}$  are the equilibrium constants of the reactions:



Since the interaction between the enzyme and the substrate,  $E + S \rightarrow ES$ , without the participation of ATP is unlikely in the presence of ATP (Whitehouse et al., 1983), formation of this complex has been neglected for simplicity. If only reactions (4) were considered, the model would predict saturation of phosphorylation (and thus of the channel activity) as ATP is increased, but would not explain the complex behavior observed experimentally, which consists of an initial increase, attainment of a maximum and subsequent decrease of the channel activity to virtually zero.

One attractive explanation for this decrease of the channel activity has been provided by a description of the very specific mechanism of interaction between the PKI and the catalytic subunit of the cAMP-dependent protein kinase. Of the fea-

tures that characterize this mechanism, the most relevant to our model are the following: (a) the participation of ATP in the formation of complexes between the inhibitor and the catalytic subunit; and (b), the finding that, for similar amounts of inhibitor and of enzyme, the percent inhibition (of phosphorylation) increased with the concentration of ATP (Whitehouse and Walsh, 1983), as if the nucleotide enhanced the ability of the enzyme to bind the peptide inhibitor.

Denoting the inhibitor by I, and postulating that this inhibitor, present together with the channel and the enzyme in the patch, is strongly bound to some component of the microenvironment of the patch, the following reaction is assumed to occur



(the interaction between the enzyme and the inhibitor without ATP [ $E + I \rightarrow EI$ ] being considered negligible). However, if only reaction (5) for binding the inhibitor were considered, some inhibition of the channel activity would indeed be predicted, but it would be difficult to account for the sharp decline in channel activity observed for ATP concentrations above 15  $\mu$ M (see Fig. 6). Similarly, if only reaction (5) is considered, this type of difficulty would be encountered in explaining the finding, using biochemical methods, that the percent inhibition of phosphorylation by the peptide inhibitor is enhanced by ATP (Whitehouse and Walsh, 1983; Van Patten et al., 1986). One way to account for the inhibitory effect of ATP within the framework of the model would be to hypothesize that enzyme-inhibitor complexes can also form with two molecules of ATP, according to the overall reaction:



Of these two binding sites proposed for ATP, one may be identified with the catalytically competent site on the kinase catalytic subunit (E). While the other site is required for fitting of the data there are no clear experimental data that indicate its existence. In the discussion this hypothetical site will be referred to as the noncatalytic site.

The treatment in the Appendix, based on the equilibria of the reactions described in this section, shows that Eq. (2) can be recast in a form which gives the explicit dependence of the channel activity on the ATP concentration, namely:

$$[\text{ch. act.}] = \frac{P[S^T]K_{AS}}{2K_{AI}} \cdot \frac{1 + \beta\gamma}{1 + K_{A_2I}[A]/K_{AI}K_A} \cdot \left\{ \sqrt{1 + \frac{4\beta}{(1 + \beta\gamma)^2}} - 1 \right\}; \quad (7)$$

where

$$\beta = \frac{K_A K_{AI}(1 + K_{A_2I}[A]/K_{AI})[E^T][A]}{1 + K_A(1 + K_{AS}[S^T])[A]} \quad \text{and} \quad \gamma = \frac{[I^T]}{[E^T]} - 1. \quad (8)$$

In Eqs. (7) and (8),  $[E^T]$ ,  $[I^T]$ , and  $[S^T]$  denote the total number of molecules of enzyme, inhibitor and substrate, that are present in the patch (complexed and uncomplexed). All the other quantities have already been defined.

Eq. (7) was used to fit the points in Fig. 6, where the channel activity in excised patches is plotted as a function of the concentration of ATP in the bath. The continuous curve is generated by Eq. (7), showing that this expression provides an

excellent fit to the complex behavior of the data. The meaning of the four parameters deduced by fitting the data with Eq. (7) is found in the footnote,<sup>1</sup> their numerical values are given in the legend of Fig. 6.

#### *Effect of Exogenously Added Inhibitor*

When some inhibitor is added to the solution bathing the excised patch, we assume that it can partition between the aqueous phase and the membrane. If  $[I_{aq}]$  is the concentration of the inhibitor added exogenously to the solution and  $[I]$  that in the membrane, we postulate a proportionality relation of the type

$$[I] = k_I [I_{aq}], \quad (9)$$

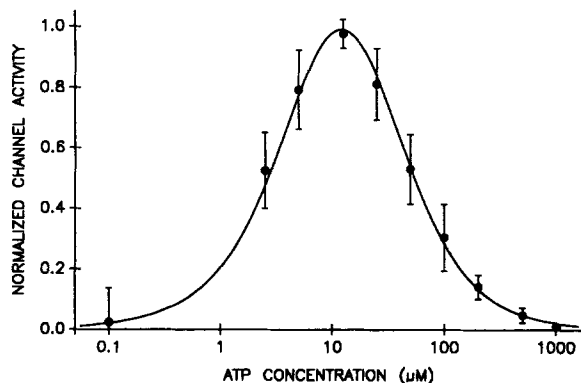


FIGURE 6. The channel activity in inside-out patches, normalized to its maximum value, is plotted vs. the ATP concentration in the bath. Channel activity is defined as the area subtended by the open-state outline of the recording trace for a given time. The data points (●) are averages from experiments in which the channel activity at the different ATP concentrations indicated were measured using the same patch. The parameters determined by fitting the data with the model (see the text) were:  $(K_{A_2I}/K_{AI}) = 5.5 \times 10^6 \text{ M}^{-1}$ ;  $(K_A K_{AI} [E^T]) = 1.4 \times 10^3 \text{ M}^{-1}$ ;  $(K_A + K_A K_{AS} [S^T]) = 10^4 \text{ M}^{-1}$ ;  $([I^T]/[E^T]) = 1.5$ .

where  $k_I$  is a partition coefficient. As is shown in the Appendix, when the inhibitor is added exogenously, the dependence of the channel activity on ATP becomes

$$[\text{ch. act.}] = P \cdot \frac{K_A K_{AS} [E^T] [S^T] [A]}{1 + K_A (1 + K_{AS} [S^T]) [A] + K_A K_{AI} k_I [I_{aq}] (1 + K_{A_2I} [A] / K_{AI}) [A]} \quad (10)$$

<sup>1</sup> $P_1 = K_{A_2I}/K_{AI}$ ,  $P_2 = K_A K_{AI} [E^T]$ ,  $P_3 = K_A (1 + K_{AS} [S^T])$ , and  $P_4 = [I^T]/[E^T]$ .  $P_1$  can be interpreted as the equilibrium constant of the reaction:  $EAI + A \rightarrow EA_2I$ . In  $P_2$ , the product  $K_A K_{AI}$  is the equilibrium constant of the reaction,  $E + A + I \rightarrow EAI$ . In the particular case that only one molecule of enzyme is present in the patch ( $[E^T] = 1$ ),  $P_2$  is then equal to that equilibrium constant. The meaning of  $P_3$  is somewhat more complex. However, if the equilibrium constant for the formation of the EAS complex (which is equal to  $K_A K_{AS}$ ) is sufficiently low,  $P_3$  is practically equal to  $K_A$ , which corresponds to the equilibrium constant for ATP binding to the enzyme, a reaction defined in Eq. (4).  $P_4$ , the ratio of the number of molecules of inhibitor to the number of molecules of enzyme in the patch, is a parameter that may play an important role in determining the channel sensitivity to ATP, as we will show later.

The difference between Eqs. (7) and (10) is due to the fact that the derivation of Eq. (10) makes use of Eq. (9), implying that the inhibitor available for interaction with the enzyme and ATP is provided by the pool, essentially unlimited, of the peptide in the bath.

The percent inhibition—defined as the ratio of the channel activity in the presence of exogenously added inhibitor to the channel activity when no inhibitor is added—is given by the ratio of Eq. (10) to Eq. (7). This quantity contains only one new parameter in addition to the four already used in Eq. (7). Using the values previously calculated by fitting the data in Fig. 6 for these four parameters, the percent inhibition as a function of the ATP concentrations is well fitted by the model. This is illustrated by the close fit of the data points with the continuous curve in Fig. 4 D.

#### *Effect of the Nonphosphorylating ATP Analogue, AMP-PNP*

In agreement with the hypothesis that the K(ATP) channel activity is coupled with phosphorylation, consistent effects of the nonphosphorylating ATP analogue, AMP-PNP, are observed only when a low basal level of ATP is also present (10–20  $\mu$ M). In this case, the analysis is slightly more complicated, since, with the two nucleotides together, the enzyme forms additional complexes, requiring the introduction of more constants for the reactions. A number of approximations were used to simplify the algebra and the fitting procedure: some because they were suggested by experimental evidence, others as reasonable approximations. They can be summarized as follows: (a) According to biochemical studies, AMP-PNP binding by the enzyme prevents further complexation with the inhibitor (Whitehouse et al., 1983, Whitehouse and Walsh, 1983). (b) Enzymatic complexes will contain two nucleotide molecules at most. Thus, denoting AMP-PNP with A\* and ATP with A, complexes (before interacting with the substrate) will be of three types: EAA\*, with the catalytically competent site occupied by ATP, EA\*A with that site occupied by AMP-PNP, and EA\*A\* with both sites occupied by AMP-PNP. Of these three complexes only the first will interact with the substrate and contribute to phosphorylation. Furthermore, since ATP is kept at a very low level, complexes with two molecules of ATP have been neglected.

Using arguments similar to those employed to deduce Eq. (2), the channel activity is given by

$$[\text{ch. act.}] = P[\text{EAS}] + P'[\text{EAA*S}], \quad (11)$$

where the first term on the right-hand side is the same as in Eq. (1), and the second represents the contribution to phosphorylation by the mixed complex containing two nucleotides and with ATP bound to the catalytically competent site of the enzyme. The second term on the right-hand side of Eq. (11) is crucial to understanding the behavior of the channel activity in Fig. 7. At low AMP-PNP levels, the increase of channel activity with the AMP-PNP concentration is due to the increasing probability of forming EAA\*S complexes. In contrast, the decline of channel activity at higher AMP-PNP levels results from a decrease of both compounds appearing in Eq. (11), consequent to the formation of complexes with two AMP-PNP molecules (EA\*A\*S) which do not participate in phosphorylation.

The explicit form of Eq. (11) was derived using the procedure outlined in the Appendix. The continuous curve in Fig. 7 shows that the model gives a very good fit to the data.

### DISCUSSION

With the introduction of the patch clamp technique it became possible to demonstrate that glucose induced membrane depolarization of the B cell is probably due to inhibition of a voltage-insensitive, inward-rectifying K channel with a conductance of 55–65 pS (Ashcroft et al., 1984). Raising ATP caused blockage of the channel in excised patches (Cook and Hales, 1984), suggesting that glucose modulates the channel activity via regulation of the intracellular ATP level (Rorsman and Trube, 1985; Misler et al., 1986; Ashcroft et al., 1987; Ribalet and Ciani, 1987).

Excised inside-out patch experiments have shown that the channel activity fre-

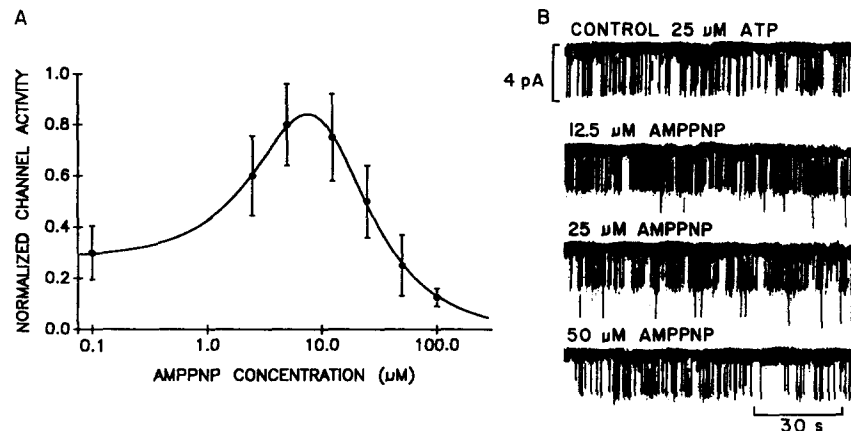


FIGURE 7. The channel activity in inside-out patches, for a fixed ATP concentration (10  $\mu\text{M}$ ), is plotted versus the concentration of AMP-PNP. The continuous curve corresponds to a fit with the model described in the text and, in more detail, in the Appendix.

quently “runs down” within 10–15 min of patch excision. This effect may be avoided by adding a low concentration of ATP (10  $\mu\text{M}$ ) (Findlay and Dunne, 1986; Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987) or GTP (100  $\mu\text{M}$ ) (Dunne and Petersen, 1986a; Findlay, 1987) to the bathing medium. In our experiments we confirmed that in some cases addition of one of these nucleotides was sufficient to slow down the decrease in channel activity, but we often found that addition of both nucleotides was necessary to keep the channel active. While the characterization of the effect of GTP is beyond the scope of this study, these data indicated that pathways other than the ATP-dependent one which is investigated here may be required to maintain the channel in an active state.

#### *Identification of the Protein Kinase Involved in Channel Regulation*

Since channel activity cannot be maintained by low concentrations of adenine nucleotides other than ATP, it has been suggested that protein phosphorylation is



required for channel functioning (Findlay and Dunne, 1986; Misler et al., 1986; Findlay, 1987; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987; Ashcroft, 1988). Intracellular injection of a specific inhibitor of protein kinase A into *Aplysia* neurons (PKI or Walsh inhibitor) alters the whole-cell K current (Adams and Levitan, 1982; Castellucci et al., 1982), suggesting that K channel regulation may be mediated by protein kinase A-dependent phosphorylation. In our study, using inside out patches, we observed that addition of either the PKI (Fig. 2) or protein kinase A regulatory subunit (results not shown) clearly inhibits the ATP-dependent K channel, implicating protein kinase A-mediated phosphorylation in maintenance of channel activity. Several important conclusions may be drawn from these findings. Since both PKI and the regulatory subunit exert their influences on the channel in the excised patch, it may be concluded that the catalytic subunit of protein kinase A is closely associated with the channel, being bound either to the patch of membrane or to components of the cellular matrix associated with it. Furthermore, the observations that the effect of ATP to maintain channel activity occurs in the absence of cyclic AMP suggest that the catalytic subunit is dissociated from the regulatory subunit in the excised patch. This suggestion is reinforced by the data obtained with PKI, since PKI can only bind to the free catalytic subunit of protein kinase A (Whitehouse et al., 1983; Whitehouse and Walsh, 1983). Extrapolating from these observations it is suggested that the regulatory subunit of protein kinase A does not remain associated with the structure close to the channel once dissociated; it is perhaps liberated into the cytoplasm and consequently lost when the patch is excised.

#### *Characterization of the Inhibitory Mechanism of Phosphorylation*

**Role of ATP.** Besides implicating protein kinase A in channel regulation, these experiments indicate a mechanistic basis to explain the dual effect of ATP on channel activity, i.e., stimulation of channel activity at low levels and inhibition at concentrations higher than 15–20  $\mu\text{M}$ . The initial, stimulatory effect of ATP is explicable simply in terms of providing the necessary phosphate groups to permit phosphorylation and therefore channel activation. To explain the effect of higher concentrations of ATP it is necessary to increase the number of components in the control system. In view of previous biochemical studies (Ashby and Walsh, 1972), and of our data illustrating the effects of the nucleotide on channel activity it is postulated that PKI or a protein with PKI-like properties may be present in the cell, or more precisely in the microenvironment of the excised patch. To account for the ATP dependence of the inhibition we further hypothesize that the binding of PKI to the catalytic subunit is ATP concentration dependent (Whitehouse and Walsh, 1983; Van Patten et al., 1986). Thus as the ATP level rises, the competition between the phosphorylation substrate and PKI for the catalytic subunit biases in favor of the catalytic subunit–inhibitor complex, the consequence of this being a fall in phosphorylation and a reduction in channel activity.

Moreover, in order to fit the observed sharp ATP concentration-dependent inhibition of the channel activity, it was necessary to assume that the enzyme–inhibitor complexes not only form with one but also with two molecules of ATP. While it seems unlikely that the catalytic subunit will have more than one binding site for ATP, it is possible that an additional molecule could bind to the inhibitor–enzyme

complex. Alternatively the steepness of the falling phase of the curve could result from the interaction of ATP with other components of the system. For instance, the channel itself may possess a nucleotide-binding site and once this site has bound ATP, reaction with the enzyme-ATP complex would still occur, but would not result in phosphorylation and channel opening.

*Inhibitory effect of exogenous PKI.* It was shown in the Results that addition of exogenous PKI in the presence of ATP significantly diminishes the channel activity. The observation that this excess inhibition due to the exogenous inhibitor is well fitted using the parameters derived from the fit of the data obtained with ATP alone suggests that ATP exerts its effect via a mechanism which includes an inhibitor of this type.

Furthermore, modeling of our data has indicated that, if the inhibitor acts as the sole regulator of kinase activity, it must be available at the site of the catalytic subunit in a similar concentration to the catalytic subunit itself. The highest concentration of PKI measured, in the studies in which such measurements have been made, indicated a concentration of ~50 nM which would give a catalytic subunit/Walsh inhibitor ratio of 10, assuming a concentration of protein kinase A of about 500 nM (Beavo et al., 1974; Demaille et al., 1977). This leads us to conclude that, if an inhibitor represents the only mechanism mediating the ATP-dependent blockage of the protein kinase effect, then either it is locally elevated at the appropriate membrane or submembrane location or protein kinase A involved in channel phosphorylation is limited to a fraction of the whole-cell kinase complement.

Since current knowledge, regarding either the concentration of the inhibitor in the B cell (Kuo, 1975) or its localization, is limited it will be difficult to judge the validity of our assumption. Resolution of the proposed mechanism of channel regulation by ATP would therefore require further biochemical studies.

*Importance of the enzyme-inhibitor ratio in determining the channel ATP sensitivity.* It has been hypothesized that channel "run-down" observed after patch excision reflects progressive loss of phosphorylation resulting from the absence of ATP. In support of this hypothesis it has been shown that run-down may be minimized when a low concentration of ATP, but not one of its nonhydrolyzable analogues, is present (Misler et al., 1986; Ohno-Shosaku et al., 1987). However, run-down may not be reversed if ATP is applied long after patch excision, suggesting that this hypothesis alone may be too simplistic. This led us to postulate that channel run-down occurs not simply because of dephosphorylation but rather, at least in part, follows the loss of one or more labile component(s) of the channel regulatory mechanism. From all the components involved in the modulation of channel activity, loss of the protein kinase A catalytic subunit would most satisfactorily explain the decay in channel activity; such a loss of catalytic subunit has been proposed to explain run-down of Ca channel activity after patch excision (Armstrong and Eckert, 1987).

Similarly, the discrepancy between the channel ATP sensitivity observed in the excised patch and that in the cell-attached patch (Ashcroft et al., 1987; Ribalet and Ciani, 1987) may be explained by a partial loss of the catalytic subunit. Since we have proposed that the channel activity is dependent on the interaction of the catalytic subunit and the kinase inhibitor, any change in the relative proportion of these two components will be reflected in an alteration of the ATP-sensitivity of channel activity. A loss of catalytic subunit in the excised patch would not only result in a

reduced ability to phosphorylate the channel (the run-down phenomenon), but by raising the ratio between kinase inhibitor and catalytic subunit it would make the blocking of the channel much more sensitive to ATP.

To test this possibility, we employed the model used previously to fit the data relating channel activity to ATP concentration in the excised patch; in this case a ratio of 3:2 had been obtained and gave a good fit of the data. In modeling the cell-attached patch situation we decreased the value of the kinase inhibitor to catalytic subunit ratio to 1:1, assuming that a fraction of the catalytic subunit was lost in the excised patch. The result of this manipulation is presented in Fig. 8 where the dotted line represents the fit obtained for the excised patch experiments (ratio 3:2) and the solid line represents the fit obtained when the ratio was lowered to 1:1 (all

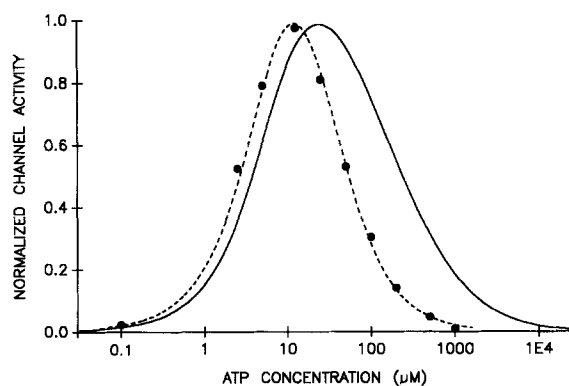


FIGURE 8. The data points and the dashed line correspond to the experimental points and the theoretical curve of Fig. 6, representing the dependence of channel activity on the ATP level. The continuous line is calculated theoretically from the model, decreasing the inhibitor/enzyme ratio,  $[I^T]/[E^T]$  from 3:2 to 1:1, but leaving all the other parameters unchanged. This simple change in the value of  $[I^T]/[E^T]$  is sufficient to shift the curve for excised patches so that the sensitivity of the channel to the nucleotide becomes similar to that deduced from experiments with intact cells and from the known intracellular concentration of ATP.

the other parameters remaining the same). As may be seen, changing the ratio from 3:2 to 1:1 caused a significant shift to the right in the sensitivity of the channel to ATP, such that 5% of the initial activity would remain in the presence of 4 mM ATP and 1% at 10 mM. By contrast, referring to the data for excised patches the activity was almost completely blocked at 1 mM ATP. These observations, based on a simple model of the interaction between ATP and the channel are supportive of the hypothesis that both rundown and the discrepancy between the ATP sensitivity of the channel in the excised and the cell-attached patch result from a loss of the catalytic subunit of the protein kinase from the excised patch.

As a corollary to this hypothesis the shift of the theoretical curves shown in Fig. 8 may be compared with the curves in Fig. 2 which represent a range of channel sen-

sitivity to ATP observed in the excise patch. Such a difference in ATP sensitivity may be explained in terms of variable levels of protein kinase A catalytic subunit remaining associated with the channels in the different patches.

*Effect of phosphorylation on channel kinetic parameters.* In all the tissues in which the ATP-dependent K channel has been studied, channel openings occur in bursts (Takei and Noma, 1984; Spruce et al., 1987; Ashcroft, 1988). This activity, characterized by groups of fast openings and closings separated by long periods of channel closure may be described by a simple model allocating one open state and two closed states to the channel. Such a model as originally proposed by Takei and Noma (1984) will be referred to in this discussion of channel kinetics. It is recognized that this represents a considerable simplification of the more complex model proposed for example, by Spruce et al. (1987), yet it is adequate for our purposes since only the slow time constants of the system were affected by the treatment applied.

As shown in Fig. 4, the addition of exogenous PKI is characterized by a dramatic increase of the long closed time intervals, giving very well-defined bursts of activity reminiscent of those observed with high concentrations of ATP. Fitting of multiple exponentials to the open and closed time histograms generated from these data confirmed that the PKI has very little effect on the fast events that take place during a burst of activity (the two fast time constants of both the open- and closed-time histograms being little affected by the inhibitor), but the PKI greatly augments the duration of the long closed times. This analysis indicates that inhibition of phosphorylation favors the occupancy of the long closed state. Conversely, manipulations that promote phosphorylation (addition of cAMP in intact cell or protein kinase in excised patches) favor the transition from the long to the short closed state, thereby shortening the duration of the closed intervals.

The comparable effect of ATP and PKI on channel kinetics (see Table I) support the hypothesis that the major effect of the nucleotide at high concentration is to favor protein dephosphorylation. This result would therefore suggest that, when the cell is exposed to glucose and the intracellular level of ATP is increased, closure of the channel is brought about by dephosphorylation.

*Characterization of the effect of nonhydrolyzable ATP analogues.* Nonhydrolyzable analogues of ATP were initially employed to test whether the channel-blocking effect of ATP involved protein phosphorylation (Cook and Hales, 1984). This hypothesis was rapidly abandoned when it was found that both AMP-PNP and AMP-PCP blocked the channel activity as efficiently as ATP, and it was suggested that both hydrolyzable and nonhydrolyzable nucleotides interact directly with the channel. Such a scheme is not compatible with the mechanism which we have proposed to explain the effect of ATP since it has been demonstrated that the interaction between the PKI and the catalytic subunit of protein kinase A occurs specifically in the presence of ATP (with  $Mg^{2+}$ ) (Whitehouse and Walsh, 1983). Consequently, one has to conclude that the inhibitory effect seen with other adenine nucleotides results from a different mechanism.

AMP-PNP and ATP bind to the nucleotide binding site of the catalytic subunit in a competitive fashion (Hixson and Krebs, 1979; Whitehouse and Walsh, 1983). In view of this observation, one may propose that once bound the nonhydrolyzable

analogues of ATP inhibit protein phosphorylation by preventing transfer of phosphate to the substrate. This mechanism is different from the one suggested for ATP which inhibits phosphorylation by promoting the formation of a catalytic subunit-inhibitor complex.

Since the formation of the catalytic subunit-kinase inhibitor complex is prevented when ATP is substituted by AMP-PNP (Whitehouse and Walsh, 1983), our model would predict that addition of AMP-PNP in the presence of an inhibitory concentration of ATP would result in partial reactivation of the channel. Reactivation would be mediated by the complex: catalytic subunit-AMP-PNP-ATP in which ATP is bound to the catalytically competent site and AMP-PNP is bound to the noncatalytic site, a configuration permitting substrate phosphorylation but not binding to PKI (see the Appendix for further details). Such reactivation was indeed observed when 5–10  $\mu\text{M}$  AMP-PNP was added to the inside of the patch in the presence of 25  $\mu\text{M}$  ATP (Fig. 7). This reversal of the ATP inhibition by the nonhydrolyzable analogue is very reminiscent of the shift in ATP sensitivity observed with ADP (Dunne and Petersen, 1986b; Kakei et al., 1986; Misler et al., 1986; Ribalet and Ciani, 1987).

AMP-PNP in excess of 10  $\mu\text{M}$  in the presence of 25  $\mu\text{M}$  ATP (Fig. 7) progressively promotes channel closure, similar to ADP in excess of 500  $\mu\text{M}$  (Misler et al., 1986; Ribalet and Ciani, 1987). We believe that this reflects progressive displacement of ATP from the catalytic subunit and the occupation of its nucleotide binding site with non-phosphate-donating nucleotides. The similarity of effects of AMP-PNP and ADP leads us to hypothesize that the well-documented effects of ADP on channel activity result from an interaction of the same type as that of AMP-PNP and the catalytic subunit. This hypothesis is supported by the observation that, as was the case with AMP-PNP, association between PKI and the catalytic subunit is not possible with ADP (Whitehouse et al., 1983).

*A role for  $\text{Mg}^{2+}$  in the control of channel phosphorylation.* Based upon these studies we have proposed that K(ATP) channel activity is determined by the degree of activation of two antagonistic mechanisms: one is the phosphorylation by protein kinase A, which stimulates channel activity; the other is the interaction of the kinase with the kinase inhibitor which results in reduced phosphorylation and therefore channel closure. In addition to the modulation of these two processes by ATP, Whitehouse and Walsh (1983) have demonstrated that both are also dependent on  $\text{Mg}^{2+}$  though with different sensitivities. Chelation of  $\text{Mg}^{2+}$  or partial substitution with  $\text{Mn}^{2+}$  (a cation that displaces  $\text{Mg}^{2+}$  from its binding site) causes a greater reduction in kinase activity than in the inhibitory effect of PKI on the kinase, these effects combining to augment the percentage inhibition of kinase activity. From these observations it may be predicted that  $\text{Mg}^{2+}$  removal at a constant level of ATP reduces K(ATP) channel activity, and indeed such has been observed in patch clamp experiments by Dunne et al. (1987). These results are consistent with our model for channel regulation, although Dunne et al. (1987) interpret the effect of lowering  $\text{Mg}^{2+}$  in terms of an increased availability of free ATP ( $\text{ATP}^{4-}$ ) to promote channel inhibition rather than a decreased protein kinase A-mediated channel phosphorylation as indicated by our model, a model which reinforces the conclusion drawn by others (Misler et al., 1986; Findlay, 1987; Ohno-Shosaku et al., 1987).

Since the role of ATP is most commonly associated with phosphorylation, it may be expected that the inhibitory effect of high ATP levels is mediated via phosphorylation rather than dephosphorylation, as demonstrated for the serotonin-sensitive K channel in the *Aplysia* neuron (Shuster et al., 1985). However, the observation that ATP and PKI affect K(ATP) channel kinetics in a similar manner suggests that the inhibitory effect of ATP results from dephosphorylation. It is acknowledged that dephosphorylation may be achieved by mechanisms different than that of the binding of an inhibitor to the catalytic subunit. For example, the regulatory subunit (type I) of protein kinase A which has properties similar to those of PKI could reassociate with the catalytic subunit if addition of ATP (which has a high affinity for this subunit) decreases the affinity of the enzyme for cAMP and consequently inactivates the enzyme (Beavo et al., 1975; Hoppe and Wagner, 1979). The fact that our experiments were also performed in the presence of ATP could have masked a possible stimulatory effect of added cAMP. Further investigation of this hypothesis is required.

Although the question of protein dephosphorylation has not been addressed in this study, the blocking effect of ATP could be explained if the phosphatase activity is regulated by ATP. Since the existence of such an ATP dependent phosphatase has been demonstrated in many tissues, and since this enzyme is, as many other phosphatases are, a multisubstrate enzyme (Merlevede, 1984), it is very plausible that dephosphorylation of the channel (or a protein associated with it) may be regulated by that enzyme in an ATP-dependent manner. However, although little is known about metabolic regulation of phosphatases because they are difficult to isolate, preliminary experiments have failed to show any evidence that cellular metabolism (for example, the level of cAMP in B cells) may affect the function of this enzyme (Sharp, 1979). For these reasons, while acknowledging that they may play a significant role in the determination of the phosphorylated state of proteins which control channel function, it is not possible to speculate further on their role. It is clear that the channel regulation by ATP is complex and while we present a model to explain the various effects of ATP it has been our intention to propose a framework for further physiological and biochemical studies rather than a definitive analysis of the mechanisms that modulate the activity of this K channel.

#### APPENDIX

##### *Model for the Effects of ATP and AMP-PNP on the Activity of the K(ATP) Channel*

*Derivation of Eq. (7).* The total number of molecules of enzyme (catalytic subunit of protein kinase A) and of inhibitor that are present in the microenvironment of the patch will be denoted by  $[E^T]$  and  $[I^T]$ , respectively. Recalling all the molecular complexes of which they are components, the equations of conservation (in the absence of AMP-PNP) can be written in the form:

$$[E^T] = [E] + [EA] + [EAS] + [EAI] + [EA_2I]; \quad (A1)$$

$$[I^T] = [I] + [EAI] + [EA_2I]. \quad (A2)$$

A similar equation should also be considered for the substrate, S, namely the channel-associated protein that is to be phosphorylated. However, the hypothesis is made that the substrate exists predominantly in its unphosphorylated state, so that

$$[S] = [S^T]. \quad (A3)$$

It is also assumed that the enzyme, the inhibitor and the substrate, which are endogenously present in the patch, cannot easily partition in water (although it is allowed that some of these molecules may be lost when the patch is excised).

Assuming that all the complexes in the right-hand side of Eqs. (A1) and (A2) are in equilibrium with their constituents, and recalling that the aqueous concentration of ATP, [A], is a known quantity, Eqs. (A1) and (A2) can be rewritten in the form of expressions containing [E] and [I] as the sole unknown variables. Considering the equilibria of reactions (3) to (6) and the approximation made in Eq. (A3), Eqs. (A1) and (A2) can be recast in the form:

$$[E^T] = [E]\{1 + K_A[A](1 + K_{AS}[S^T] + K_{AI}[I]) + K_{A2}[A]^2[I]\}; \quad (A1')$$

$$[I^T] = [I]\{1 + [A][E](K_A K_{AI} + K_{A2}[A])\}. \quad (A2')$$

Eq. (7), whose derivation is the purpose of this part of the Appendix, is obtained by eliminating [I] from the two equations above, solving for [E] and substituting the result, together with the right-hand side of Eq. (A3), in Eqs. (3) and (2) of the Results section.

*Derivation of Eq. (10).* When the peptide inhibitor is added exogenously, Eq. (A2) or, equivalently, Eq. (A2'), may be replaced by the much simpler Eq. (9), implying that the number of free inhibitor molecules in the patch is kept constant by exchange with the large supply of that molecule in water. Substituting the right-hand side of Eq. (9) in Eq. (A1'), the latter can be solved for [E]. Similarly to the case of Eq. (7), Eq. (10) is obtained by substituting this explicit expression for [E] in Eqs. (3) and (2).

*Method for deriving the dependence of the channel activity on the AMP-PNP concentration, Eq. (11).* When ATP is partially substituted with AMP-PNP, the system becomes more complicated owing to the greater number of molecular aggregates that may form. For example, denoting AMP-PNP with [A\*], compounds of the type EA\* and EA\*S must be considered in conjunction with the corresponding ones with ATP, requiring the introduction of the new equilibrium constants,  $K_A^*$  and  $K_A^*S$ . However, compounds of the type EA\*I are neglected, since experimental evidence indicates that AMP-PNP binding by the enzyme strongly inhibits further interaction with the inhibitor (Whitehouse et al., 1983; Whitehouse and Walsh 1983). Despite the variety of possible molecular aggregates, it was not possible to describe the experimental dependence of the channel activity on the AMP-PNP concentration assuming a simple one-to-one stoichiometry for enzyme-nucleotide binding. However, an excellent fit to the data could be obtained assuming that, as the AMP-PNP concentration is increased, the complexes EAS and EA\*S can still bind a molecule of the nucleotide according to the reactions:



(Equally satisfactory results would be obtained considering different pathways: e.g., the one whereby two nucleotide molecules would bind to the enzyme before interaction with the substrate, although this scheme would require the introduction of more types of complexes, and thus more parameters).

As it has already been pointed out in the Results [see Eq. (11)], both complexes in Eq. (A4) are involved in phosphorylation. The explicit dependence of [EAS] and [EAA\*S] on the ATP and the AMP-PNP concentrations is derived after a procedure entirely similar to that adopted in the first section of this Appendix: using the conservation equations for E and I, an

expression is deducted for [E], which is then substituted in Eq. (3) to obtain [EAS], as well as in the equation of chemical equilibrium for reaction (A4) to obtain [EAA\*S]. Finally, the expressions for [EAS] and [EAA\*S] as functions of the concentrations of ATP and AMP-PNP are substituted in Eq. (11) to deduce the channel activity as functions of the same variables.

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